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Investigations in Enhancing the Reproducibility of Implantable Amperometric Glucose Biosensors for Mass Production

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Table of Contents:

Chapter 1: Introduction

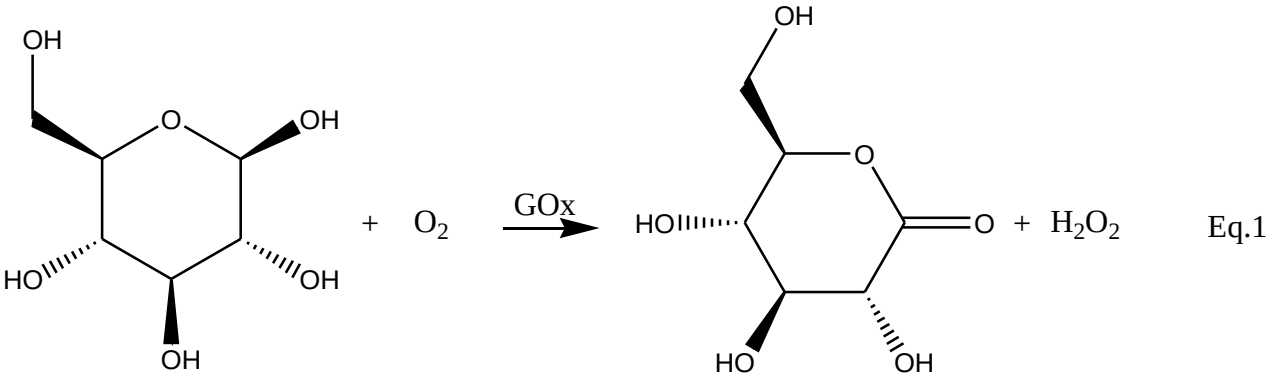
Diabetes mellitus is a metabolic disorder which results in the body's inability to regulate blood glucose. It is estimated that 347 million people live with this disorder worldwide[1]. There are two types of diabetes, type I diabetes is characterized by deficient insulin production whereas type II the body's insensitivity to insulin. If left untreated, diabetes increases the risk of developing complications such as retinopathy, nephropathy, and neuropathy[2]. Therefore it becomes vital for diabetics to regulate their blood glucose level to decrease their risk of developing these complications. The conventional method of monitoring blood glucose level involves pricking the finger and drawing blood onto a test strip. However, this is a very inconvenient way to monitor blood glucose. An alternative method would be to us an implantable glucose sensor that would continuously monitor blood glucose levels and transmit the data to a proximal receiver eliminating the frequent painful process of prinking the fingers.

Various techniques have been employed in fabrication of these glucose sensors including electrochemical, optical, near-infrared, Raman, fluorescence, and piezoelectric technology with the common goal of creating stable and reliable sensors[3-6]. However, the Clark-type electrochemical sensors have become the popular choice in fabricating these sensors due to its use of glucose oxidase giving high specificity to d-glucose[7-9].

1.1 Background

In 1962, Clark and Lyons from the Children Hospital of Cincinnati were the first to propose the idea of having an enzyme based glucose sensor[10]. Their first device utilized an oxygen electrode covered by semipermeable dialysis membrane entrapping a thin layer of

glucose oxidase (GOx) monitoring the consumption of the O_2 catalyzed by the enzyme according to the following reaction:



ß-D-glucose D-glucono-1,5-lactone Eq.1

In 1975, Yellow Spring Instrument Company made further advances to these Clark sensors by making a glucose analyzer capable of measure glucose in 25µL sample of blood[7]. Updike and Hicks made further developments to these sensors by using two oxygen working electrodes; one covered with immobilized GOx and the other to measure the differential current in the same sample for correcting the background variations of oxygen in the sample[11]. In 1973 the first amperometric glucose sensor was introduced by Guilbault and Lubrano which measured the hydrogen peroxide (H_2O_2) produced in Eq.1 instead of the oxygen (O_2) consumed by immobilizing GOx on an electrode using cellophane and applying an electrical potential[12]. An applied potential of 0.5V results in the following reaction (Eq.2) producing a current proportional to the glucose concentration in the sample:

$$H_2O_2 \xrightarrow{0.5\,V} O_2 + 2H^+ + 2e^- \qquad \text{Eq. 2}$$

Since then, a wide variety of amperometric glucose sensors have been developed varying in electrode design or material, membrane composition, or immobilization method[7]. Despite the advances, when these sensors are placed in the body and interact with its complex physiology, numerous problems start to rise. Under physiological conditions, oxygen becomes the limiting

reagent saturating GOx. Enzyme saturation is of concern when discussing glucose sensors because it prevents the sensor from detecting increasing glucose concentration beyond the saturation point. This is because the concentration of oxygen in blood is much lower than that of glucose. In normal to hyperglycemic conditions, this difference can be up to one to two orders of magnitude[13].

A second problem associated with amperometric glucose sensors is the specificity of the response current produced to glucose. Other endogenous species such as ascorbic acid and uric acid and exogenous species such as acetaminophen are oxidized at the same applied potential as H_2O_2 (0.5 V versus Ag/AgCl) eliciting an increased current thus suggesting higher than the actual glucose concentration[7,13]. Another problem arises with the implantation of the sensor resulting in a body response which leads to biofouling, inflammation and eventual fibrosis[14]. This creates a problem by hindering both glucose and oxygen from getting to the sensor where GOx is located eventually leading to the degradation of sensor sensitivity to glucose[15]. This will lead to decreased life time of the sensor ultimately resulting in the need to change the sensor more often.

Another concern with these sensors is their linearity. The current produced should be proportional to the concentration of glucose in the sample but due to the saturation problem, the current produced is less than expected as the concentration of glucose is increased. In addition to that, increased sensor response time is of concern because of the mass transfer limitations[6].

1.2 UConn's Glucose Sensor Design

To address the aforementioned problems associated with amperometric glucose sensors, the UConn team has developed an optimized sensor design based on layer stratification[16]. The

first layer that is put on the Pt working electrode is a thin electropolymerized polyphenol (PPh) layer. This addresses the issue of specificity by preventing species with large molecular weight such as uric acid (168.11 g/mol), ascorbic acid (176.12 g/mol), and acetaminophen (151.17 g/mol) from reaching the working electrode and getting oxidized without affecting the ability of H_2O_2 (34.0147 g/mol) to diffuse through the layer reaching the electrode and getting oxidized. Moving outward from the working electrode, the second layer consists of GOx which is immobilized via cross-linking by glutaraldehyde. Next, the sensor is dip coated with polyurethane (PU), the third layer, resulting in a uniform 3µm film on top of the GOx layer. PU coating has widely been used due to its biocompatibility and its glucose-diffusion limiting behavior in glucose sensor[17]. Since it decreases the amount of glucose getting to the GOx and not O_2 (the limiting reagent in Eq. 1), this increases the oxygen-to-glucose ratio surrounding the GOx layer preventing GOx from quickly getting saturated, and rendering the sensor linear within physiological glucose concentrations between 2 to 22mM[18].

In addition to serving as a glucose diffusion limiting barrier, the PU membrane also prevents the outward diffusion of GOx-generated H_2O_2 from the GOx layer. The presence of GOx drives the inward diffusion of both glucose and O_2 allowing them to get through the PU membrane. However it is solely the permeability of the PU membrane that influences the outward diffusion of H_2O_2. In order to maintain good sensor sensitivity and decrease sensor response time, it is important to prevent the building up of H_2O_2 by increasing the outward diffusion of GOx-generated H_2O_2[19-20]. This is because having too much H_2O_2 (product of GOx catalyzed reaction, Eq. 1) surrounding the enzyme will decrease the turnover number (k_{cat}) increasing the sensor response time and decreasing the sensitivity. To address this issue, a thin layer of glutaraldehyde-immobilized catalase (the fourth layer) is added on top of the PU

membrane to serve as an additional driving force for the outward diffusion of H_2O_2. This layer accomplishes this by catalyzing the conversion of H_2O_2 into O_2 as shown by Eq. 3 below increasing the outward diffusion of the GOx-generated H_2O_2 through the PU membrane, and preventing the decrease in k_{cat} of GOx. Because the k_{cat} of catalase is 40 times greater than that of GOx, this provides and effective way of removing H_2O_2 from layer 2[21]. In addition, this also provides two additional benefits. For one, it decreases the risk of possible tissue damage done by H_2O_2 leaking out of the sensor[22]. And secondly, it produces O_2 which as mentioned earlier is a limiting reagent in Eq. 1 thus preventing quick saturation of GOx.

$$2H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O \quad \text{Eq. 3}$$

The fifth and final layer added on top of the catalase layer is a poly(vinyl alcohol) (PVA) hydrogel matrix which is cross linked in place following application through three repetitive freeze-thaw cycles. Upon freezing, water begins to form microcrystals causing partial PVA dehydration[23]. This dehydration leads to the formation of microspheres that act as physical cross links giving the sensor mechanical support, and protecting the inner layers during the sensor implantation[23]. Additionally, these microspheres can be used as host for a variety of drugs that prevent tissue response such as inflammation caused by the puncture of the tissue during the implantation of the sensor[24]. These microspheres degrade slowly over time thus providing sustained release of the drug controlling unwanted tissue response for a longer period of time[24]. The addition of this layer does not affect the continuous flow of glucose and O_2 into the sensor and the outward diffusion of byproducts such as H_2O_2 and therefore does not affect sensor performance[23, 25]. Figure 1 below depicts the cross section of these 5-layer sensors.

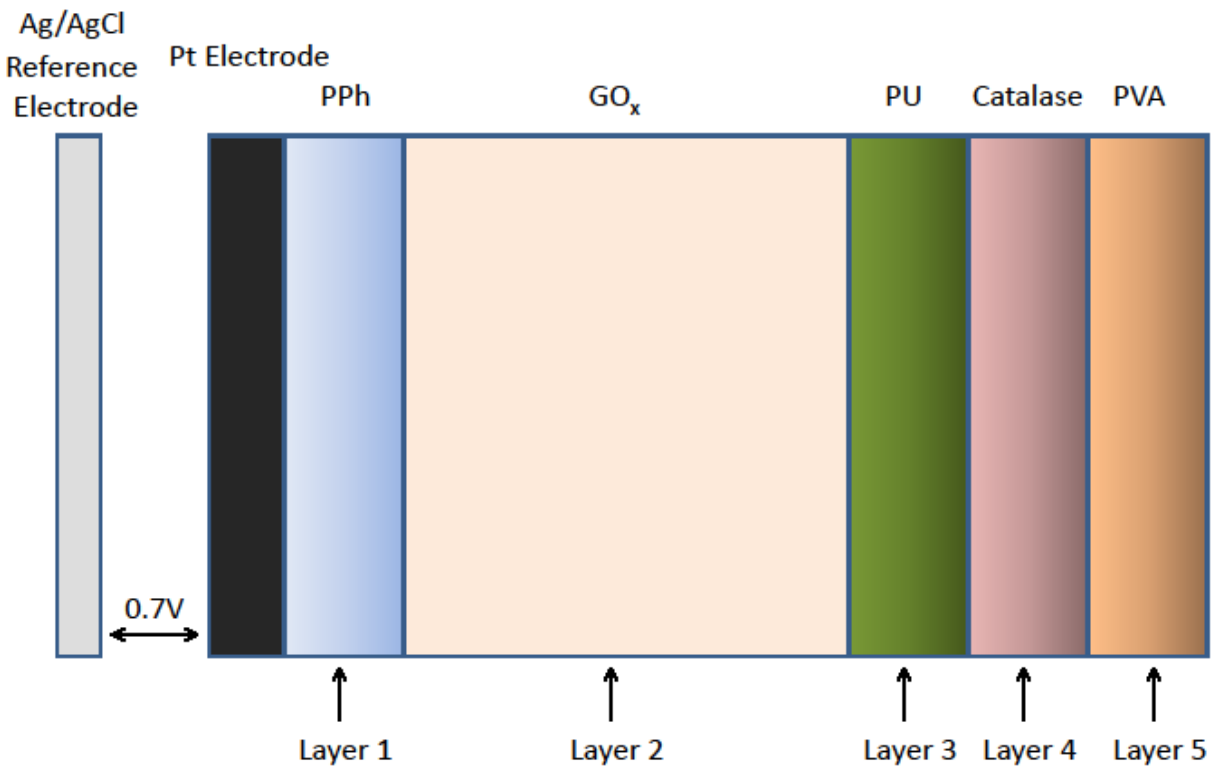


Figure 1: The above figure is a schematic representation of the cross section of UConn's 5-layer glucose sensor architecture. (layer thickness in the figure is not according to scale)

1.3 Objective

In light of complex design described above, the large scale production of these 5-layer sensors is compromised by the lack of high sensor-to-sensor reproducibility. Herein, we examine the reproducibility of the sensor after the application of each layer to investigate the origin of sensor-to-sensor irreproducibility.

Chapter 2: Materials & Methods

2.1 Materials

The following lists all of the materials used in this study:

1. 125 µm platinum (Pt) & silver (Ag) wires purchased from World Precision Instruments
2. Electrochemical analyzer (Model CHI1030A Series Multi-Potentiostat)

The following materials were purchased from Sigma-Aldrich:

3. 10 mM Hexachloroplatinic acid (H_2PtCl_6) made from 99.9% Hexachloroplatinic acid hydrate
4. 100 mM Phenol was made from phenol loose crystals (MW 94.11)
5. 4% (w/w) Polyurethane (PU) solution in 98% tetrahydrofuran (w/w) & 2% dimethylformamide (w/w).
6. Glucose oxidase enzyme (GOx) (E.C. 1.1.3.4, 157,500 units/g, Aspergillus niger)
7. Bovine serum albumin (BSA)
8. Catalase (E.C. 1.11.1.6, 5000 U/mg)
9. 25% (w/v) Glutaraldehyde aqueous solution made from grade 1: 50% (w/v) glutaraldehyde aqueous solution
10. 1 M d-glucose made from reagent grade d-glucose

2.2 Experimental Methods

Fabrication of the Coil-Type Glucose Sensor:

The coil type glucose sensor was constructed by coiling a 125 µm Pt wire which will serve as the working electrode. The working electrode was then followed by the reference electrode in close proximity to the working electrode by coiling a 125 µm Ag wire. Once the sensor was constructed physically, the Ag/AgCl reference electrode was made on the surface of the silver wire by placing the device in a stirring solution of 0.3 M HCl and applying a constant potential of 0.7 V vs. a saturated calomel electrode placed in the same solution for 400 seconds. The electrodes were then subsequently rinsed with distilled water and let to dry in air.

The surface area of the working electrode was then electrochemically cleaned in a 0.5 M sulfuric acid (H_2SO_4) solution via potential cycling between -0.21 and 1.19 V for 21 cycles. The device was then rinsed by dipping it in distilled water for 3 seconds. Next, platinum nanoparticles were electrochemically deposited onto the working electrode by submerging it in 10 mM H_2PtCl_6 in 0.1 M hydrochloric acid (HCl) and applying a constant potential of -0.3 V vs. the Ag/AgCl reference electrode on the sensor itself for 300 seconds.

Once the sensor was ready for its first layer, phenol was added to the working electrode by putting the device in a 100 mM phenol solution and applying a constant potential of 0.7 V vs. the Ag/AgCl reference electrode on the sensor for 2100 seconds. The end result of this step was a thin electropolymerized layer of polyphenol on top of the working electrode. The next layer, GOx, was then put on the device by dip-coating the PPh-coated working electrode in a solution of GOx, BSA and glutaraldehyde. The glutaraldehyde slowly forms cross-links that immobilize the enzyme.

Continuing on with the sensor fabrication, a layer of polyurethane was then put on the device by dipping it in 4% (w/w) PU solution in 98% tetrahydrofuran (w/w) & 2% dimethylformamide (w/w). A thin layer of catalase was then added to the sensor by dipping it in a solution of catalase, BSA, and glutaraldehyde in the same manner as the GOx layer. The PVA layer was not added to the batches of sensors used in this study because reproducibility of the PVA layer has previously been studied by this group[16].

In vitro Amperometric Experiments:

After the addition of each layer, and prior to the construction of the first layer, the sensor is tested *in vitro* by amperometric experiments in a stirred phosphate-buffered saline (PBS) solution (pH ≈ 7.4) maintained at 37°C for glucose tests and room temperature for H_2O_2 tests. A CHI1030A Series Multi-Potentiostat was used to apply a constant potential of 0.5 V vs. an Ag/AgCl reference electrode. Sensor response current versus various glucose/H_2O_2 concentrations was measured by raising the concentration every 100 second following an initial 500 second background stabilization period.

Production of Batches:

Sensors were made following the aforementioned design in a batch process. All experiments were done on all of the sensors in the batch together, i.e., layer 1 was added to all sensors in batch 1 at the same time and tested for H_2O_2 following the application of the layer simultaneously in independent cells. 4 batches of sensors were made with each batch containing 10 sensors each.

Performance Analysis:

The sensitivity of the test was determined by obtaining the slope of the linear range of the sensor response current versus the glucose/H_2O_2 concentration. To calculate the relative standard deviation (RSD) between sensors, the following formula was used:

$$\% \, RSD = \frac{Average}{standard \; deviation} \, x \, 100$$

Chapter 3: Results and Discussion

3.1 Variations in the Sensitivity of the 4 Layer Glucose Sensors

Data analysis of both batches 1 (B1) and 2 (B2), which contained the 4 layers under investigation led to the generation of Figure 2. As illustrated in the figure, the application of the PPh layer yielded the highest RSD value out of 4 the layers, 46% and 48% for batch 1 and 2, respectively. The RSD value of the remaining layers was lower than that of PPh. Additionally, it can be seen that the greatest change in RSD occurred after the application of the PPh layer with a change of 38% and 39% for batch 1 and 2, respectively. The application of the remaining layers resulted in either a decrease of RSD or an insignificant increase for both batches.

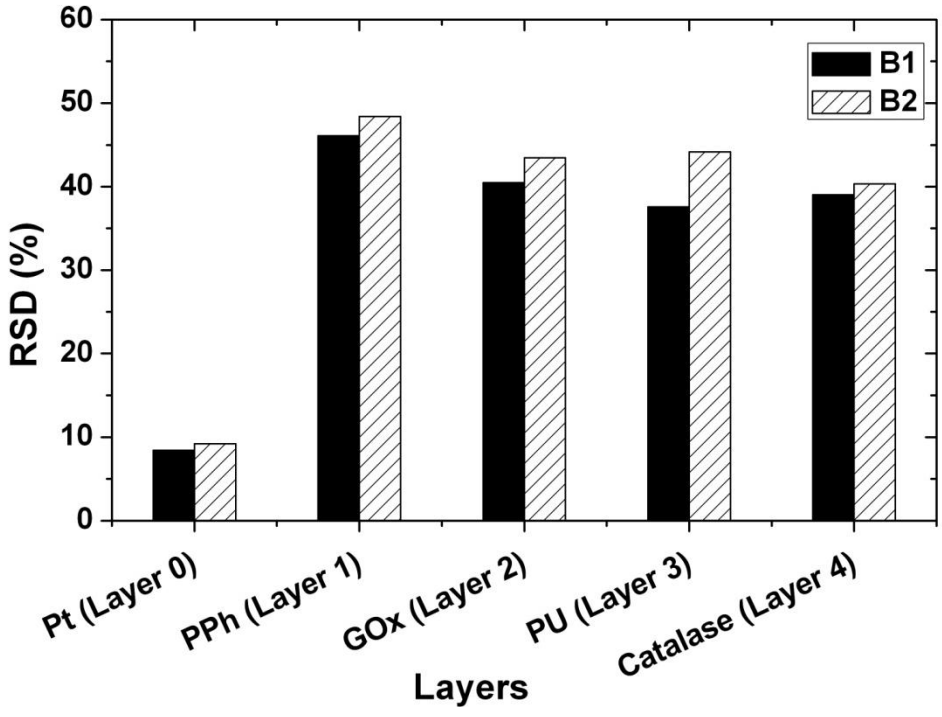


Figure 2: This figure shows the RSD values before the application of the first layer and after the application of each layer thereafter up to catalase. It can be seen that the application of PPh results in the greatest RSD out of all the layers.

Since the PPh was electropolymerized onto the sensor by applying a constant potential with an electrochemical analyzer, the current produced by each sensor as a function of time for 2100 seconds was obtained from the CHI file. The RSD value of the current was then calculated for both batches at each second. Both batches showed a similar pattern: RSD increased as time went on. Figure 3 below shows the data obtained from the CHI. Batch 1 and 2 yielded an average RSD value of 128% and 295% respectively for the current produced by the sensors in their batch. At the start of the 2100 second period, the RSD of the current produced was 42% and 196% for batch 1 and 2 respectively. As time went on, the RSD value of the current for both steadily increased ending at 159% and 298% for batch 1 and 2 respectively at 2100 seconds.

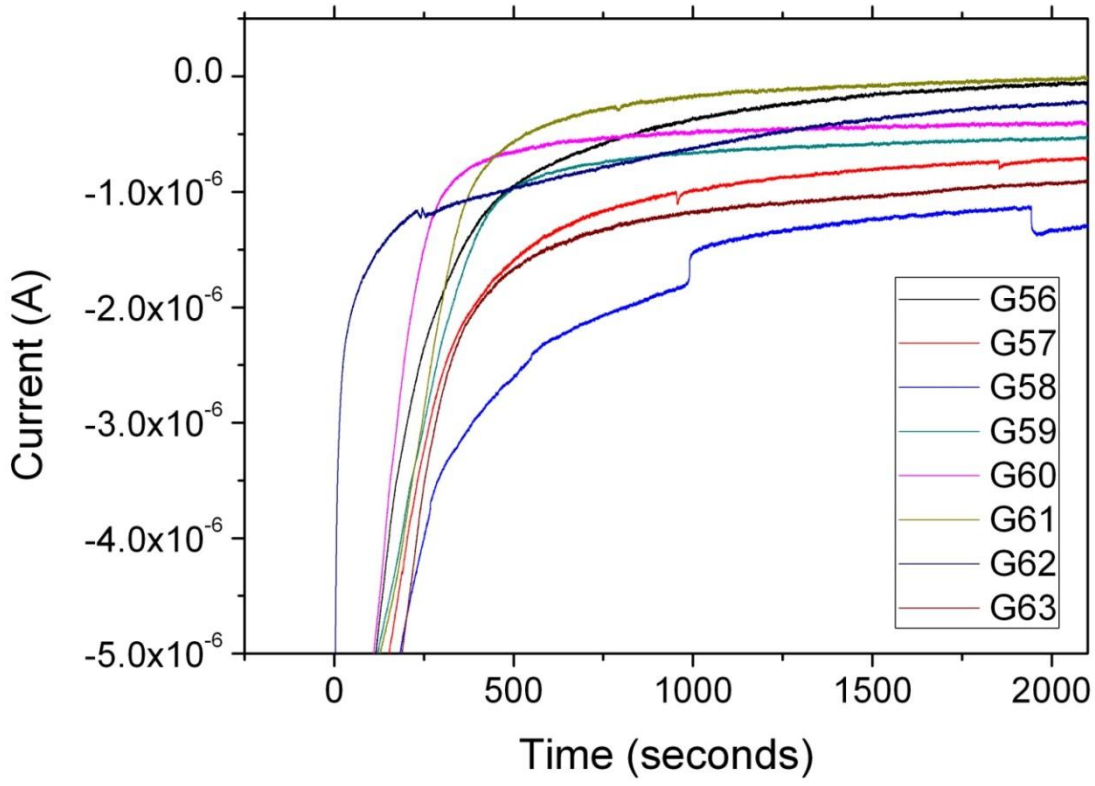


Figure 3: The figure above shows the data obtained from the CHI file during the electropolymerization of PPh onto the sensors of batch 1. It can be seen that there exists a greater variation towards the completion of the 2100 second period than towards the start of the PPh electropolymerization.

The variations in current obtained from the CHI file suggests a non-uniform electropolymerized layer being deposited on to the sensor surface. Figure 4 below models a possible explanation for this variation. Figure 4a shows all of possible sites for polyphenol electropolymerization once the sensor is immersed into the PPh solution. However, once the constant voltage is applied, instead of electropolymerizing the PPh uniformly, it is only occurring at random sites resulting in a non-uniform PPh layer as shown in Figure 4b. This ultimately results in high sensor-to-sensor irreproducibility because it is occurring at random and not uniformly throughout the sensor. In theory, however, the applied voltage should be forming a uniform layer of electropolymierzed PPh onto the working area of the sensor as illustrated in Figure 4c.

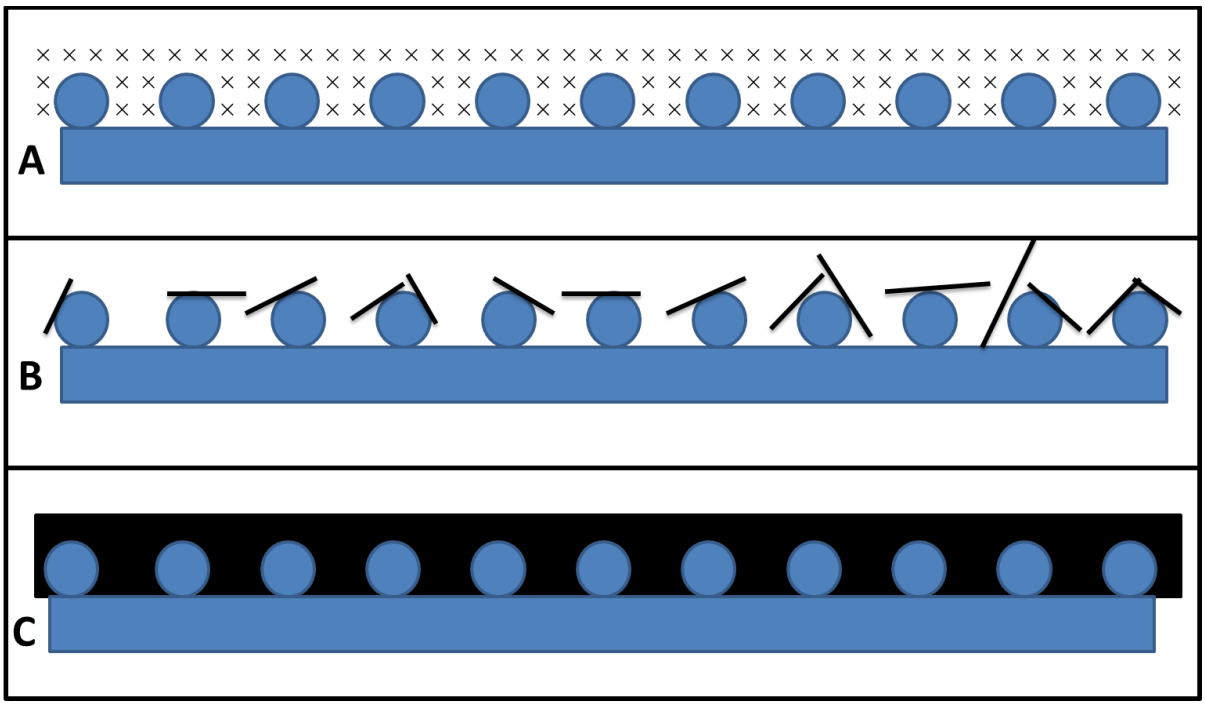


Figure 4: The figure above is a schematic representation of the surface area of the working electrode where the blue represents the working electrode itself. The crosses in panel A represent all of the possible sites where electropolymerization of PPh is possible and should occur. The lines in panel B represent the non-uniform electropolymerization on the surface of the pt working electrode. The shaded dark region around the Pt working electrode in panel C represents the theoretical uniform electropolymeriation of the PPh layer.

3.2 Variations in the Background Current of the 4 Layer Glucose Sensors

Following the 500 second stabilization period, prior to the addition of the first species either H_2O_2 or glucose, there is a background current that varies from sensor to sensor. Figure 4 was generated by calculating the RSD values of the background current at each layer under investigation for batches 1 and 2. It can be seen that the electropolymerization of PPh results in the highest sensor variation of the background current (164% in batch 1 and 160% in batch 2) out of all the layers. This most likely is the outcome of the high variations in the current generated during the electropolymerization of PPh as discussed above in Figure 3. Beyond that, a decreasing pattern is seen with the addition of each layer ending with 50% and 39% for batch 1 and 2 respectively at the catalase level.

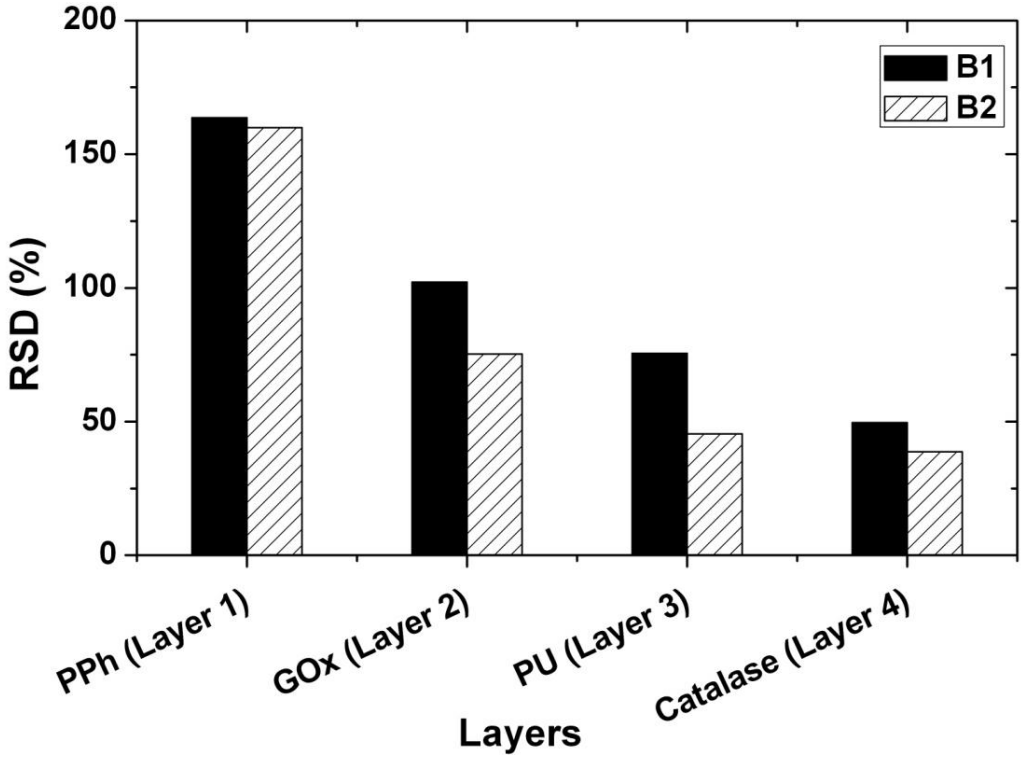


Figure 4: The figure above plots the RSD seen in the background current in batches 1 and 2 at each layer under investigation. A general decreasing trend can be seen from the PPh with the highest variation in background current occurring at the PPh layer.

3.3 Variations in the Sensitivity of the 3 Layer Glucose Sensors (No PPh)

Since the data analysis of both batch 1 and 2 suggested that the PPh layer is the source of greatest irreproducibility, batches 3 and 4 were made without this layer. As illustrated by Figure 5 below, the RSD following the application of the 3 layers was 24% maximally after the application of the GOx layer.

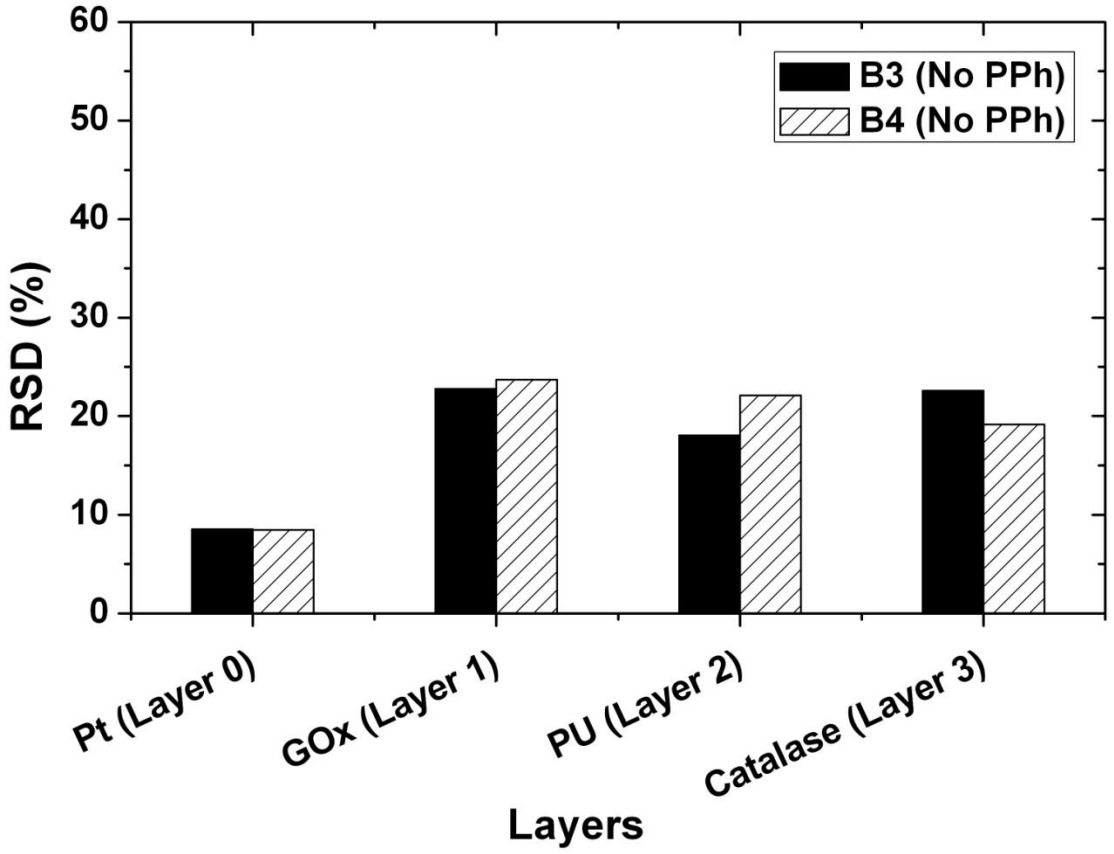


Figure 5: This figure plots the RSD of batch 3 and 4 that were fabricated without the PPh layer. Unlike batches 1 and 2, there is not a dramatic increase in RSD after the application of the first layer (PPh in the case of B1 and B2, and GOx in the case of B3 and B4).

Plotting the RSD values of the batches of sensors with and without PPh generated Figure 6. Figure 6a compares batch 1 and 4 because they have the lowest RSD prior to the application of the first layer (PPh in the case of batch 1 and GOx in the case of batch 4), whereas Figure 6b

compares batch 2 and 3 because they have the highest RSD prior to the application of the first layer. As can be seen by both figures, the RSD is significantly lower for the batches without the PPh layer giving an RSD value around 20% at each layer compared to 40% at each level with batches with the PPh layer.

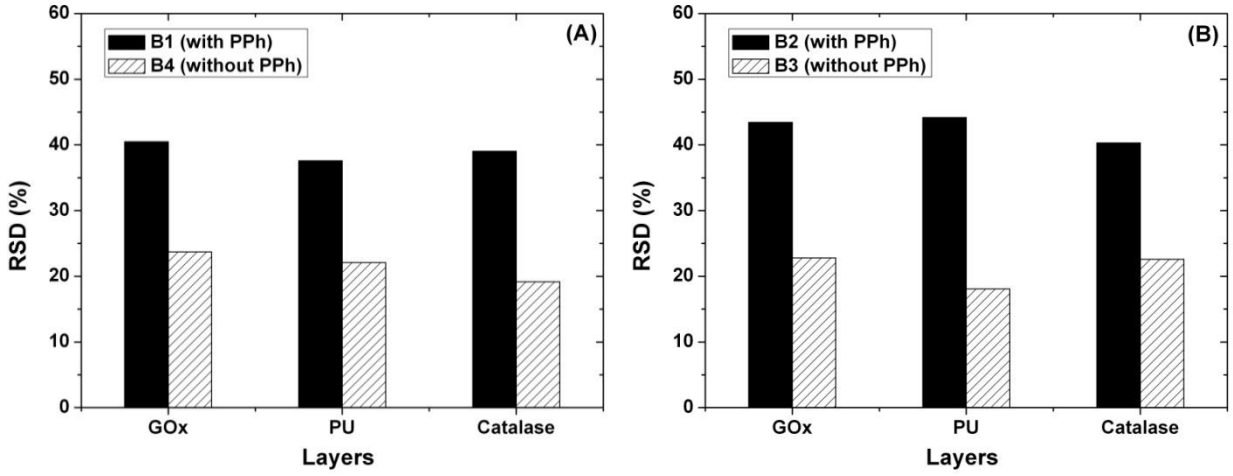


Figure 6: The figure above compares the RSD at the GOx, PU, and catalase layers of two types of sensors: with and without the PPh layer. It can be seen that in both Figure 6a and 6b that the batch of sensors without the PPh layer had significantly less variation compared to the batch of sensors with the PPh layer.

Chapter 4: Conclusion

The 5-layer glucose sensor architecture composed of (1) PPh, (2) GOx, (3) PU, (4) catalase, (5) PVA work collectively to address the various problems associated with the first-generation Clark-based electrochemical glucose sensors. However, the large scale production of these 5-layer sensors is compromised by the lack of high sensor-to-sensor reproducibility. Two batches of sensors (with 10 sensors in each batch) were fabricated according to the above design up to but not including PVA because previous studies addressing the reproducibility of the PVA layer, since that study has been conducted before. Before the application of the first layer and after the application of each later thereafter, the sensors were tested for their sensitivity to assess their reproducibility to determine the origin of sensor-to-sensor irreproducibility. Both batches suggested that the PPh layer contributed the greatest amount to sensor-to-sensor irreproducibility with the highest RSD in both batches at this level. Furthermore, the variation in the background current was also assessed at each level for these batches of sensors. As anticipated, the background current varied the greatest after the application of the PPh layer possibly for the same reason that affected the reproducibility in sensitivity of these sensors.

To further confirm this, 2 more batches were fabricated without the PPh layer. The RSD at the catalase layer decreased from 39% and 40% for batches 1 and 2 respectively to 23% and 19% for batches 3 and 4 respectively. This indicates that the PPh layer is in fact the greatest source of irreproducibility to the glucose sensor architecture discussed in this paper.

Chapter 5: Future Outlook

At the conclusion of this project, we determined that the source of irreproducibility for the 5-layer glucose sensor to be the PPh layer in terms of sensitivity. Our future experiments include standardizing the surface area of the working electrode with the sensitivity of the sensors. Due to the fact that these coil-type glucose sensors are made by hand, the surface area from sensor-to-sensor will vary. The greater the surface area, the more H_2O_2 can be oxidized producing a greater current that results in an increased sensitivity of the sensor to the species. In the future, we hope to standardize the sensitivity of each sensor by dividing it by the area of the working electrode.

Additionally, we hope to move away from these handmade coil-type sensors to automatically constructed sensors either coil-type or non-coil type to give the least amount of RSD in the surface area of the working electrode to start with.

Another possible study to be conducted is finding the most reproducible method to incorporate the PPh layer into the sensor without causing such high sensor-to-sensor irreproducibility because of its importance in increasing the specificity of the sensor to glucose. Studies can be done to optimize the electropolymerization time of phenol or concentration of the phenol solution used during the process. Additionally, a new technique can be utilized to deposit the PPh on the sensor. Instead of polymerizing the phenol by applying a constant potential, a thin layer of PPh can be sprayed onto the sensor using the spin coating method to obtain a uniform layer.

Chapter 6: References

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